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Hydroxyproline-rich glycoproteins accumulate in pearl millet after seed treatment with elicitors of defense responses against *Sclerospora graminicola*

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The accumulation of hydroxyproline-rich glycoproteins (HRGPs) was investigated after induction of resistance in pearl millet against downy mildew caused by *Sclerospora graminicola*. Treatment of susceptible pearl millet seeds with various biotic and abiotic elicitors resulted in increased HRGP content in the cell walls of coleoptiles at 9 h after inoculation. Similar results with increased accumulation at 4–6 h after inoculation were obtained in suspension cells of pearl millet. Maximum HRGP accumulation was observed in seedlings raised from susceptible seeds treated with chitosan and *Pseudomonas fluorescens*. Western blot analysis with MAC 265 (a rat monoclonal antibody raised against pea HRGP) identified three proteins of 27, 17 and 14 kDa in resistant cultivars. The absence of the 14 kDa HRGP was observed in susceptible cultivars as reported earlier. The induced accumulation of the 14 kDa HRGP upon elicitor treatments was observed in the present study. Peroxidase and hydrogen peroxide, essential components for HRGP cross-linking, were also increased in samples treated with these elicitors. A tissue specific increase in HRGP at the regions around vascular bundles was observed upon chitosan treatment. The results presented will have a presumed importance in identifying the susceptible pearl millet varieties and improving those using elicitors of defense for field applications.

1. Introduction

Success of a plant defense response depends on the speed by which the plant recognizes the attacking pathogen and the intensity by which appropriate defense responses are activated. The basal resistance response in plants to restrict the colonization of the pathogen can be enhanced by specific biotic and abiotic stimuli in the form of elicitors [1–4]. Protection of pearl millet [*Pennisetum glaucum* (L.) R. Br] against the downy mildew causing oomycete *Sclerospora graminicola* (*Sacc.*) *Schroet* is possible by application of abiotic elicitors such as β-amino butyric acid (BABA) [5], proline [6], chitosan [7], Trichoshield [8] and 2,6-dichloroisonicotinic acid (DCINA) [9]. It has also been shown that microorganisms like *Pseudomonas fluorescens* [10] and plant extracts of *Datura metel* [11,12] have the potential to control *S. graminicola*.

The effect of abiotic and biotic elicitors involves biochemical changes in the host metabolism that may play a role in limiting plant infection by *S. graminicola*. Cell wall reinforcements due to accumulation and cross-linking of hydroxyproline-rich glycoproteins (HRGPs) as a response to *S. graminicola* has been reported [13]. HRGPs are important plant cell wall structural components, which during the course of pathogen invasion are induced in several plant pathogen interactions [13–16]. The involvement of HRGPs in systemic acquired resistance (SAR) has been established recently using transformed tobacco cultivars having the *nahG* gene for salicylate hydroxylase. The transformed plants that were insensitive to salicylic acid signaling showed poor HRGP accumulations [15]. Also a highly co-ordinated localized alteration to plant cell walls with HRGP accumulation was show at the challenge sites of pathogen infection using monoclonal antibodies specific to HRGPs [13,17]. This represents a rapid defense mechanism to strengthen

Abbreviations: HRGPs, hydroxyproline-rich glycoproteins; Hyp, Hydroxyproline; hai, hours after inoculation.

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the cell wall as a barrier to pathogen ingress prior to the development of transcription dependent defenses [18].

The possible mechanism by which HRGP accumulation contributes to disease resistance involves cross-linking between HRGP monomers catalyzed by peroxidase and hydrogen peroxide to form a network, which might provide anchorage for lignifications and creates a barrier impenetrable to fungal hyphae [16,18]. The current study was carried out to investigate the role of HRGPs during the induction of resistance in pearl millet against *S. graminicola* by seed treatment with selected biotic and abiotic elicitors.

2. Materials and methods

2.1. Plant material

Pearl millet cultivars 7042S (highly susceptible, HS) with >25% downy mildew disease incidence (DMDI) and IP18296 (highly resistant, HR) with 0% DMDI after inoculation with *S. graminicola* under field conditions were used in the study. The seeds were obtained from the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Hyderabad, India. The seeds of each line were sown in the downy mildew disease plot of the Department of Studies in Biotechnology, University of Mysore, Manasagangotri, Mysore 570006, Karnataka, India, for testing their reaction to the disease following the procedure of Williams et al. [19].

2.2. Pathogen and preparation of inoculum

S. graminicola was isolated from pearl millet cv. 7042S and maintained on the same cultivar under greenhouse conditions and was used for all inoculation experiments. Leaves of infected plants showing symptoms of downy mildew were collected in the evening, washed in running tap water to remove the remnants of previous sporulation, blotted dried, cut to pieces about 2 inches in length and placed in a moist chamber for sporulation. Fresh sporangia were collected the next morning and zoospores released by them used as inoculum [20].

2.3. Test seedlings used for the study

Seeds of resistant cv. IP18296 and susceptible cv. 7042S cultivars of pearl millet were surface sterilized in 0.1% sodium hypochlorite for 15 min and washed thoroughly with sterile distilled water. Seeds of the susceptible cv. 7042S were treated with the biotic and abiotic elicitors. The concentrations of elicitors used and duration of treatments were chosen based on earlier studies (Table 1). For each elicitor treatment, one hundred seeds were used. Simultaneously, seeds of the resistant and susceptible cultivars were treated with distilled water under similar conditions to serve as a standard control of resistance.

The treated and the untreated/standard control seeds were further germinated on moist filter paper under aseptic conditions at 25 ± 2 °C in darkness for two days. The two-day-old seedlings were inoculated by the root dip technique with a 4×10^4 zoospores ml^{-1} suspension of *S. graminicola* [20]. Seedlings dipped in sterile distilled water served as an uninoculated control. The seedlings were harvested at 8/9 h after inoculation for further experiments.

2.4. Analysis of hydroxyproline-rich glycoproteins (HRGPs)

2.4.1. Hydroxyproline (Hyp) content in cell walls of pearl millet coleoptiles

Test seedlings from resistant, susceptible and elicitor treated susceptible seeds were sampled at 9 hai (hours after inoculation) with *S.graminicola*. Seedlings dipped in sterile distilled water served as an uninoculated control. Cell walls from the coleoptiles regions of the test seedlings were isolated following the procedure of Shailasree et al. [13]. The coleoptiles of the seedlings were homogenized using pestle and mortar at 4 °C in 0.5 M potassium phosphate buffer, pH 7.0. The complete disruption of cells in the paste was examined by light microscopy. The homogenized suspension was centrifuged at 10,000*g* for 10 min. The pellet obtained was repeatedly washed with buffer followed by distilled water for five times. Washed cell walls were suspended by vigorous stirring in 5 volumes of 1:1 (v/v) chloroform–methanol. The organic solvent was carefully removed with out disturbing the cell wall pellet. Cell walls were washed three times with 5 volumes of acetone and then air-dried. The amount of HRGPs was determined by analyzing the Hyp content in the cell wall hydrolysate. Hydrolysis of the cell walls took place with 6 N HCl for 18 h at 110 °C in sealed tubes. Hydrolysates were evaporated to dryness. Hyp was then extracted in the minimum amount of distilled water from the dried hydrolyzed samples and the amount estimated following the spectrophotometric method of Prockop and Udenfriend [21]. Hyp content was expressed as μg Hyp mg^{-1} cell wall (dry weight).

2.4.2. Hydroxyproline in suspension cells of pearl millet

The pearl millet cell culture was raised from the susceptible (7042S) cultivar by following the method of Vasil and Vasil [22]. The well-established suspension cells were regularly sub-cultured onto fresh medium at 1:5 dilution rates at 10-day intervals and after 10 sub-cultures the cells were used for the study. A cell culture (10^8 cells ml^{-1}) at the mid-point of log phase of growth (16 day old) was used for the experiment. The suspension cells were treated with elicitors *P. fluorescens* (UOMSAR 14) at 10^8 cfu/ml or Chitosan

Table 1
List of biological and chemical elicitors selected for HRGP accumulation study. The different concentrations of the elicitors used for the seed treatments in the present study and the treatment time are indicated in the table. The similar treatment gave a field protection, to the susceptible cv. 7042S against *S. graminicola* as reported from the references indicated.

Elicitor	Concentration	Time of seed treatments	Field protection observed	Reference
Chitosan (Sigma)	0.3% in distilled water	9 h	73%	Sharathchandra et al. [7]
2,6 dichloroisonicotinic acid (INA)	0.2 mM in distilled water	6 h	73%	Shivakumar et al. [9]
Pseudomonas fluorescens (UOMSAR– 14)	10^8 cfu/ml^{-1}	6 h	70%	Raj et al. [10]
Trichoshield[a]	5% in distilled water	6 h	67%	Raj et al. [8]
Datura metel	2% leaf extract in distilled water	3 h	67%	Devaiah et al. [11] Shivakumar et al. [12]
Proline	15 mM in distilled water	3 h	67%	Raj et al. [6]

[a] A talc-based formulation containing 100 million spores per gram of *Trichoderma harzianum*, *Gliocladium virens*, and *Bacillus subtilis*, was obtained from Nutri-Tech Solution P/L. Queensland, Australia.

(Sigma, St. Louis, USA), at 0.3% in distilled water for 1 h. After treatment the suspension cells were inoculated with zoospores of *S. graminicola* (4×10^4 spores ml^{-1}) and harvested at different time intervals, viz., 0 h (before inoculation with the pathogen), 2, 4, 6, and 8 h after inoculation. After washing thoroughly in distilled water, cell walls were extracted from the suspension cells and Hyp content was determined as detailed in subsection 2.4.1.

2.5. Extraction of total cell wall proteins

Test seedlings from resistant, susceptible and chitosan/*P. fluorescens* treated susceptible seeds were sampled at 9 hai with *S. graminicola*. Seedlings dipped in sterile distilled water served as an uninoculated control. Cell wall proteins were extracted from coleoptiles of the seedlings as reported by Shailasree et al. [13]. All procedures were carried out at 4 °C. Coleoptiles were homogenized in 0.5 M potassium phosphate buffer, pH 7.0, followed by centrifugation at 10,000*g* for 10 min. Subsequently, the suspension was washed five times with the same buffer followed by washing with distilled water. The pellet was suspended in three volumes of 3:1 (v/v) absolute ethanol: 1.25 N HCl and incubated at 4 °C. After two days, cellular debris was removed by centrifugation at 10,000*g*. Proteins were precipitated by adding 3 volumes of cold acetone followed by incubation at 4 °C overnight. The precipitated proteins were centrifuged at 10,000*g* for 15 min. Acetone was decanted and the pellet was air-dried.

2.6. Electrophoresis

Total protein from the cell wall extracts were separated by sodium dodecyl sulphate- polyacrylamide gel electrophoresis (SDS-PAGE) following the method of Laemmli [23] in a 1 mm thick, 12% polyacrylamide gel. The acetone precipitate was dissolved in 0.05 M sodium acetate buffer (pH 3.5). Fifty microgram protein equivalents of each sample were loaded into the gel. Following SDS-PAGE, separated proteins were stained with Coomassie blue. Glycoproteins in the total cell wall extract were identified by periodic acid Schiff (PAS) staining [13].

2.7. Western blot analysis

Immediately after SDS-PAGE, gels were blotted onto nitrocellulose membranes (Millipore) using a Multiphor II (LKB, Pharmacia) electrophoretic transfer apparatus according to the manufacturer's protocol. The blots were blocked in 2% fat-free milk powder in Tris buffered saline (TBS: 10 mM Tris HCl, pH 8.0, 150 mM NaCl). The blots were incubated for 2 h at 37 °C with primary antibodies (MAC 265, a rat monoclonal antibody against pea HRGP [24], kind gift from Elizabeth A. Rathbun, John Innes Centre, England) diluted in TBS buffer. After washing three times with TBS, the blots were incubated with anti-rat IgG horseradish peroxidase-conjugate for 1 h at room temperature followed by three washes with TBS. Subsequently, the blots were stained for peroxidase activity with 1.33 mM 3,3′-diaminobenzidine (DAB, Sigma, MO, USA) and 10 mM hydrogen peroxide. The proteins on the blots were quantified using the Bioprofile Image System (Vilber Lourmat, France). Results are presented in arbitrary units.

2.8. Tissue printing

Test seedlings from resistant, susceptible and chitosan treated susceptible seeds were sampled at 9 hai with *S. graminicola*. Tissue print was carried out as described by Cassab and Varner [25]. Coleoptile regions were separated and cross-sectioned, dried on a kim wipe, and pressed onto nitrocellulose membrane for 30 s. Nitrocellulose paper was pretreated with 0.2 M $CaCl_2$ for 30 min and dried before use. After printing the paper was air-dried for 10 min and subjected to immunolabeling. The blots were blocked in 3% BSA in Tris buffered saline (TBST: 10 mM tris (pH 7.2), 0.8% NaCl and 0.05% Tween 20) for 1 h. The blots were probed with MAC 265 monoclonal antibody as described above in the western blot analysis. The images were observed using a stereo binocular microscope (Wild Heerbrugg, Switzerland) with high magnification and recorded using a digital camera (Nikon coolpix 990) attached to the microscope.

2.9. Peroxidase activity and isoforms

Test seedlings from the resistant, susceptible and chitosan/*P. fluorescens* treated susceptible seeds were sampled at 8 hai with *S. graminicola*. Seedlings dipped in sterile distilled water served as an uninoculated control. The peroxidase activity and isoforms accumulation pattern was obtained and compared in these samples.

2.9.1. Extraction of protein

Seedlings were harvested 8 hai and coleoptiles of the seedlings homogenized in 2 ml of 0.05 M phosphate buffer, pH 7.0, at 4 °C and centrifuged at 12,000*g* for 15 min. The supernatant was used as crude enzyme for spectrophotometric assay of peroxidase and isoelectric focusing (IEF) analysis. The protein concentration was determined by the dye binding method of Bradford [26] using bovine serum albumin as standard (Sigma, St. Louis, USA).

2.9.2. Spectrophotometric analysis of peroxidase activity

Peroxidase assay was carried out as described by Hammerschmidt et al. [27]. The reaction mixture (3 ml) consisted of 0.25% (v/v) guaiacol and 10 mM hydrogen peroxide in 10 mM potassium phosphate buffer, pH 6.9. Addition of 5 μl of crude enzyme extract initiated the reaction, which was measured spectrophotometrically at absorbance (A_{470}) (Hitachi U 2000, Japan). Peroxidase activity was expressed in terms of change in A_{470} for the linear phase of the slope (A_{470} min^{-1} mg^{-1} protein). Results are presented from individual experiments, with 25 seeds per treatment. Three independent experiments were performed.

2.9.3. Isozyme analysis of peroxidase using isoelectric focusing (IEF)

IEF was performed on a 1.5 mm, 7.5% polyacrylamide gel containing 2% ampholyte (pH 3–10, Sigma, St. Louis, USA) using a Multiphor II (LKB) system according to the manufacturer's protocol. p*I* markers (Sigma) ranging from p*I* 3.6 to 9.3 were co-electrophoresed to estimate the p*I* of the proteins. Forty micrograms of protein were loaded at the center of the horizontal gel maintained at 2 °C. IEF was performed at 2 °C for 3 h by stepwise increases in voltage: 200, 400, 600, and 800 V for 30 min each and lastly 1000 V for 1 h. After electrophoresis, gels were stained according to the method of Schrauwe [28]. The p*I* of the peroxidase isozymes were calculated using the Image Analysis System (Vilber Lourmat, France). The isoenzymes showing differential accumulation were quantified using the Bioprofile Image System (Vilber Lourmat, France). Results are presented in arbitrary units.

2.10. Localization of H_2O_2

Test seedlings from resistant, susceptible and chitosan treated susceptible seeds were sampled at 8 hai with *S. graminicola*. Seedlings dipped in sterile distilled water served as an uninoculated control. Coleoptile peelings from the test seedling were used for H_2O_2 localization following the method of Thordal-Christensen et al. [29]. The peelings were placed in freshly prepared solutions of 1 mg ml^{-1} of 3,3′-diaminobenzidine (Sigma, St. Louis, USA), pH

3.8 at 26 °C. After incubation for 30 min, the epidermal peelings were washed with 96% ethanol and mounted in 10% glycerol for light microscopy. H_2O_2 was seen as dark brown coloration in the cell walls. They could be classified into the following categories viz, 0) no accumulation; 1) light and confluent accumulation; 2) dark and patchy accumulation.

3. Results

3.1. Accumulation of HRGPs in pearl millet as a response to treatment with various biotic and abiotic elicitors

Several biotic and abiotic elicitors that are reported to protect pearl millet against *S. graminicola* infection (Table 1) were investigated for their ability to induce cell wall reinforcement through HRGPs. The accumulation of HRGPs as determined by Hyp content in the cell walls of pearl millet coleoptiles at 9 hai is presented in Fig. 1. Treatment of seeds with elicitors and further challenge inoculation with *S. graminicola* resulted in increased amounts of Hyp. The maximum level of Hyp in uninoculated plants was observed in the resistant cv. IP 18296 (0.28 μg Hyp mg^{-1} cell wall, dry weight) and this increased after inoculation to 0.53 μg Hyp mg^{-1}. The Hyp content in the control of the susceptible cv. 7042S was significantly lower (0.16 μg Hyp mg^{-1}) and it did not change after inoculation. Treatment of the susceptible cultivar with chitosan or *P. fluorescens* resulted in increased constitutive Hyp content (0.21 μg Hyp mg^{-1}). Inoculation with *S. graminicola* increased the Hyp accumulation to 0.41 and 0.45 μg Hyp mg^{-1} in chitosan and *P. fluorescens* treated plants, respectively. Seed treatment with proline, INA, *D. metel* and trichoshield did not result in significant increase in Hyp concentration compared to susceptible untreated control. However these treatments followed by further inoculation with *S. graminicola* resulted in increased Hyp content to 0.27, 0.36, 0.34 and 0.27 μg Hyp mg^{-1} for proline, INA, *D. metel* and trichoshield treatment, respectively (Fig. 1).

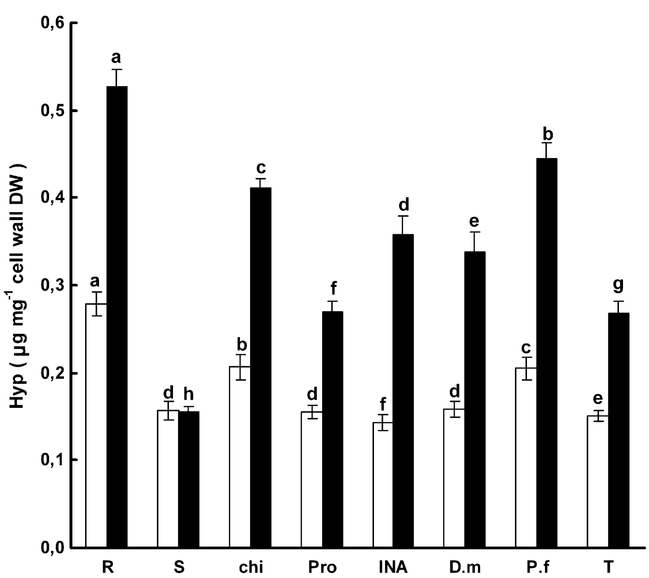

Fig. 1. The (Hydroxyproline) Hyp accumulation in the coleoptiles of pearl millet seedlings. The surface sterilized seeds of susceptible cv.7042S were treated with different elicitors (100 seeds per treatment). The untreated cv.7042S and resistant cv. IP18296 were used as standard controls of resistance. The elicitor treated and the untreated seeds were germinated on moist filter paper under aseptic conditions at 25 ± 2 °C in darkness for two days. One set of both treated and untreated seedlings germinated were root dip inoculated with *S. graminicola*. The other set was processed as uninoculated controls. The Hyp was estimated in all the samples collected and compared. The samples are pearl millet seedlings uninoculated control (□) and inoculated with *S. graminicola* (■). R: Resistant pearl millet cultivar; S: Susceptible pearl millet cultivar; Chi: Chitosan treated susceptible; Pro: Proline treated susceptible; INA: 2,6-dichloroisonicotinic acid treated susceptible; D.m *Datura metel* treated susceptible; P.f: *Pseudomonas fluorescens* treated susceptible and T: Trichoshield treated susceptible. The data are means of three independent experiments. Bars indicate ± SE. Means designated with the letter are not significantly different according to Tukeys HSD test at $P < 0.05$.

Since significant increase in HRGP accumulation was observed only for chitosan and *P. fluorescens* treatments, these were selected for further studies. Pearl millet suspension cells were established and a time course study on the accumulation of Hyp was carried out in susceptible and elicitor treated susceptible variety of pearl millet. The Hyp content remained constant in control samples. One hour treatment of suspension cells with chitosan and *P. fluorescens* resulted in the increased accumulation of Hyp in cell wall extracts of suspension cells. Maximum accumulation level was observed at 6 h after inoculation with *S. graminicola* in elicitor treated cells (Fig. 2A and B).

3.2. Analysis of acid-ethanol extracted proteins and identification of HRGPs

Cell wall proteins extracted from the coleoptiles of seedlings raised from seeds of resistant, susceptible and elicitor treated susceptible cultivars (9 hai with *S. graminicola*) were analyzed by electrophoresis. Distilled water treated seedlings were kept as a control check. Coomassie blue staining of SDS-PAGE separated proteins revealed several bands with molecular weights ranging from 45 to 14 kDa (Fig. 3A). To identify glycoproteins, PAS staining of the SDS-PAGE gel was carried out. A 17 kDa stained for PAS in all the samples (Fig. 3B). Western blot analysis using MAC 265 identified 27, 17 and 14 kDa HRGP in resistant cultivars (Fig. 3C). MAC 265 antibody revealed that the 14 kDa band absent in the uninoculated samples of susceptible cultivar was induced upon elicitor treatments (Fig. 3C). The two major proteins of 17 and 14 kDa reacted with higher intensity in resistant and elicitor treated susceptible (chitosan and *P. fluorescens*) samples upon inoculation with the pathogen.

3.3. Tissue printing

Tissue printing and immunolabeling with MAC 265 antibody showed differential localization of HRGPs in the cross sections of coleoptiles from all test samples of pearl millet seedlings (Fig. 4). An intense HRGP accumulation was observed in resistant cultivar of pearl millet specifically in the regions of vascular bundles which further increased upon challenge inoculation with *S. graminicola* at 9 hai (Fig. 4A). The susceptible cultivar did not show any intense banding pattern for HRGP during the same time interval (Fig. 4B). Interestingly, scattered and increased accumulation of HRGP was observed around the vascular bundles of chitosan treated susceptible cultivar upon challenge inoculation (Fig. 4C). Tissue print analysis of uninoculated samples revealed very limited staining (results not shown) indicating absence of these structural defenses.

3.4. Peroxidase assay

Peroxidase activity was determined in coleoptiles of resistant, susceptible, as well as in the chitosan/*P. fluorescens* treated pearl millet samples (Fig. 5). The maximum constitutive peroxidase activity was observed in IP18296 and this increased significantly 8 hai with *S. graminicola*. On the other hand, peroxidase activity was not significantly altered in the susceptible cultivar after inoculation. Treatment of susceptible seeds with chitosan and *P. fluorescens* resulted in marginal increased peroxidase activity of the seedlings

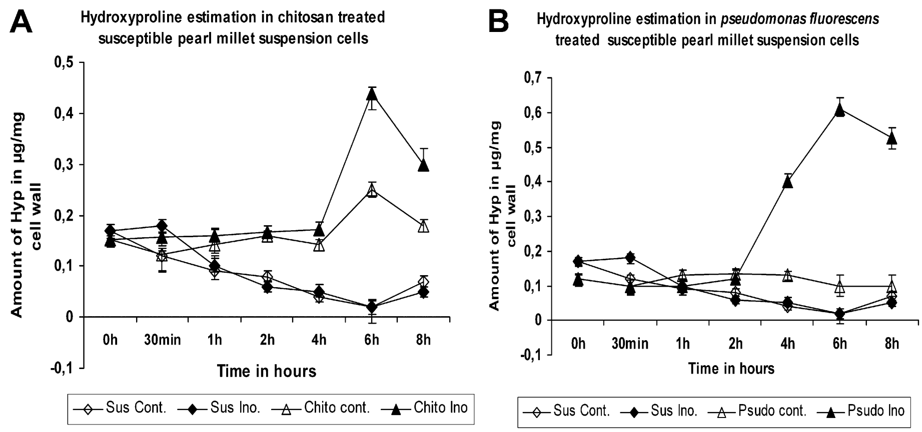

Fig. 2. The Hyp accumulation in elicitor treated suspension cells of susceptible cv. 7042S. (2A) Chitosan treatment was carried out for 1 h. The samples were further inoculated with a suspension of *S. graminicola* and collected at different time intervals. Distilled water treated suspension cells were kept as a control check. The samples are: (◇) Susceptible control; (◆) Susceptible inoculated; (Δ) Chitosan treated control and (▲) Chitosan treated suspension cells inoculated with *S. graminicola*. (2B) *P. fluorescens* treatment was carried out for 1h. The samples were further inoculated with suspension of *S. graminicola* and collected at different time intervals. Distilled water treated suspension cells were kept as a control check. The samples are: (◇) Susceptible control; (◆) Susceptible inoculated; (Δ) *P. fluorescens* treated control and (▲) *P. fluorescens* treated inoculated. The values are means of three independent experiments. Bars indicate ± SE.

compared to the control. This activity increased significantly after inoculation with the pathogen.

3.5. Peroxidase isoform analysis by isoelectric focusing (IEF)

Peroxidase isoforms were separated by IEF and detected by in-gel activity staining. Several basic and acidic isoforms were seen (Fig. 6A). Of these, the basic isoforms corresponding to p*I* 8.9, 8.7 and 8.5 stained with higher intensity in elicitor treated samples compared to their respective controls. Quantification of these bands also indicated higher accumulation of these isoforms in the samples treated with elicitors compared to the untreated ones (Fig. 6B).

3.6. Analysis of H_2O_2 localization

The accumulation of H_2O_2 was assessed by the appearance of brown coloration within the periplasmic space of seedling tissue after staining with DAB. The hypersensitive response (HR) lesions are visible microscopically as brownish-black spots. H_2O_2 accumulation was evaluated at 8 hai with the pathogen in the epidermal peelings of test seedling coleoptiles. The accumulation was observed in all test seedlings, but to varying degrees. In case of resistant cultivar HR like reaction showing the accumulation of H_2O_2 within cells close to the parasite (haustoria) was observed upon *S. graminicola* inoculation at 2 h (Fig. 7A). With increase in time interval, a dark and confluent H_2O_2 deposition was observed

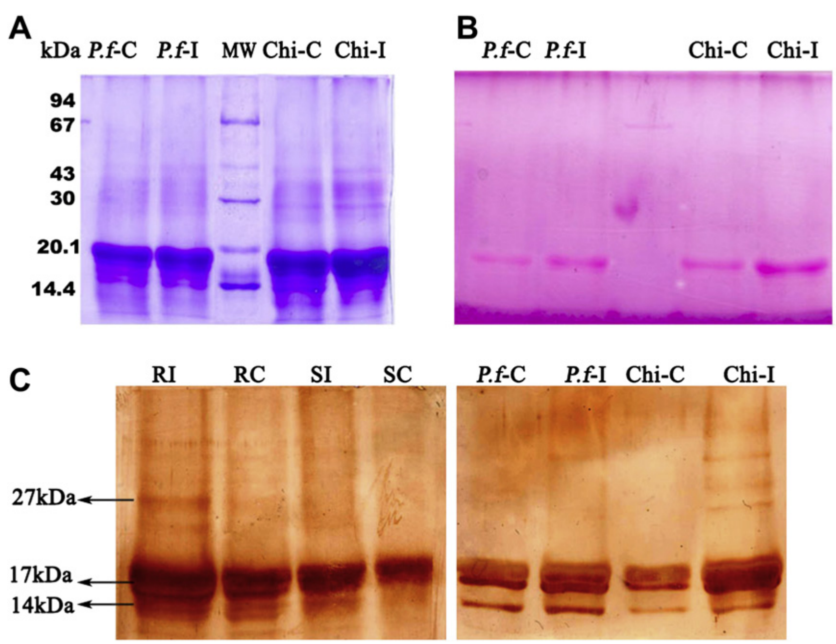

Fig. 3. Analysis of acid-ethanol extracted proteins, HRGPs identification and induction pattern obtained (A) Coomassie blue; (B) Periodic acid Schiff staining and; (C) Western blot analysis using the MAC 265 antibody [24] of total cell wall proteins extracted from coleoptiles of the resistant (IP18296) and susceptible (7042S) and elicitor treated susceptible pearl millet cultivar. RC: Resistant control; RI: Resistant inoculated with *S. graminicola*; SC: Susceptible control, SI: Susceptible inoculated with *S. graminicola*; *P.f* C: *P. fluorescens* treated susceptible control; *P.f* I: *P. fluorescens* treated susceptible plants inoculated with *S. graminicola*; Chi-C: chitosan treated susceptible control; Chi-I: chitosan treated susceptible plants inoculated with *S. graminicola*; MW: low molecular weight markers. (For interpretation of the references to colour in figure legends, the reader is referred to the web version of this article.)

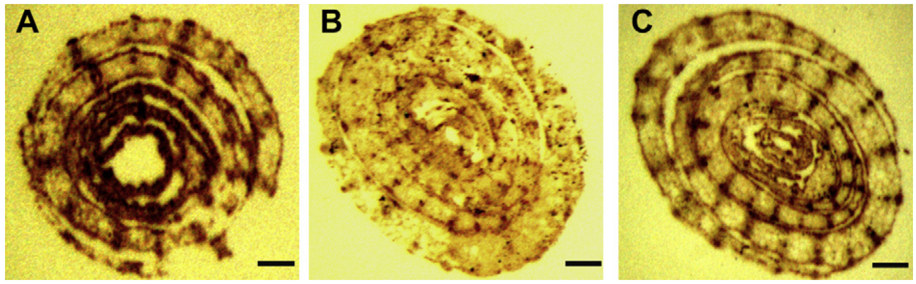

Fig. 4. Tissue print immunoblot localization of HRGPs. Cross sections of two day old- resistant, susceptible and chitosan treated susceptible pearl millet seedlings challenged inoculated with *S. graminicola* were used. The prints made on a nitrocellulose membrane were immunolabeled using MAC 265 monoclonal antibody (1:100 dilution) and DAB stained in the presence of H_2O_2. A: Resistant; B: Susceptible; C: Chitosan treated susceptible. Bars 50 µm.

(Fig. 7B). In seedlings of the susceptible control, H_2O_2 accumulation was light and confluent (Fig. 7C) and this changed to small, dark and patchy spots 8 hai with *S. graminicola* (Fig. 7D). When chitosan was used as an elicitor, H_2O_2 accumulation was induced as evidenced by its more pronounced dark and confluent appearance in susceptible cv. upon 8 hai with *S. graminicola* (Fig. 7E).

4. Discussion

The present study investigated the induction pattern of HRGPs in a susceptible cultivar of pearl millet following treatment with several biotic and abiotic elicitors. The accumulation of HRGPs was determined by monitoring the Hyp content in the cell walls. The colorimetric estimation of Hyp is reported to be a sensitive indicator for the presence of HRGPs [30]. Results of the present study indicated a four fold increase in Hyp in the cell walls of resistant pearl millet cv. IP18296 upon *S. graminicola* inoculation, when compared to susceptible cv.7042S. The analysis of Hyp among seedlings raised from susceptible seeds treated with abiotic and biotic elicitors indicated an increase in the wall-bound HRGP level upon elicitor treatment. Furthermore, when the Hyp accumulation (Fig. 1) was compared to the downy mildew protection data (Table 1), a higher amount of Hyp was recorded in those treatments where the protection against *S. graminicola* exceeded 70% under field conditions. Among the various elicitors used in the study, induction of Hyp was observed more prominently in chitosan and *P. fluorescens* treated samples. These treatments showed a further three fold increase in Hyp accumulation during challenge inoculation with *S. graminicola* when compared to the susceptible controls. Higher accumulation was observed after 6 h of inoculation with *S. graminicola* in the elicitor treated suspension cells of susceptible pearl millet cultivar. These results indicate that the seed treatment with

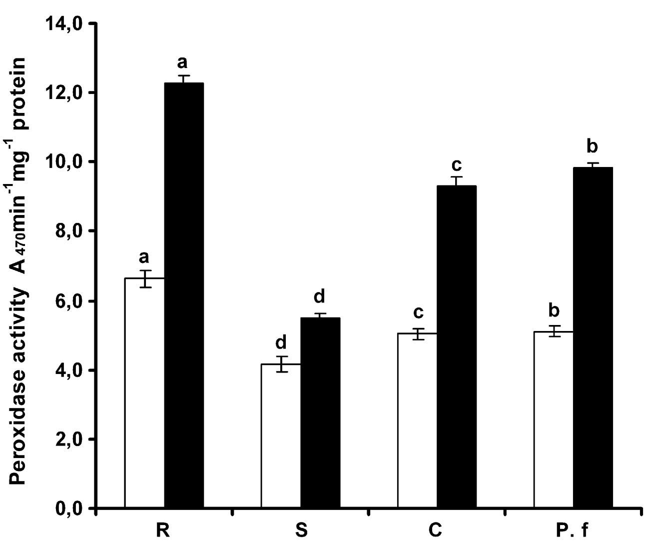

Fig. 5. Total peroxidase activity in coleoptile extracts of a resistant cv. IP18296, susceptible cv. 7042S and elicitor treated susceptible pearl millet seedlings. The samples are control (□) and inoculated 8 with *S. graminicola* (■) samples of pearl millet. R: Resistant cv.; S: Susceptible cv.; C: Chitosan treated susceptible cv.; P.f. *Pseudomonas fluorescens* treated susceptible cv. The data are means of three independent experiments. Bars indicate ± SE. Means designated with the same letter are not significantly different according to Tukeys HSD test at $P < 0.05$.

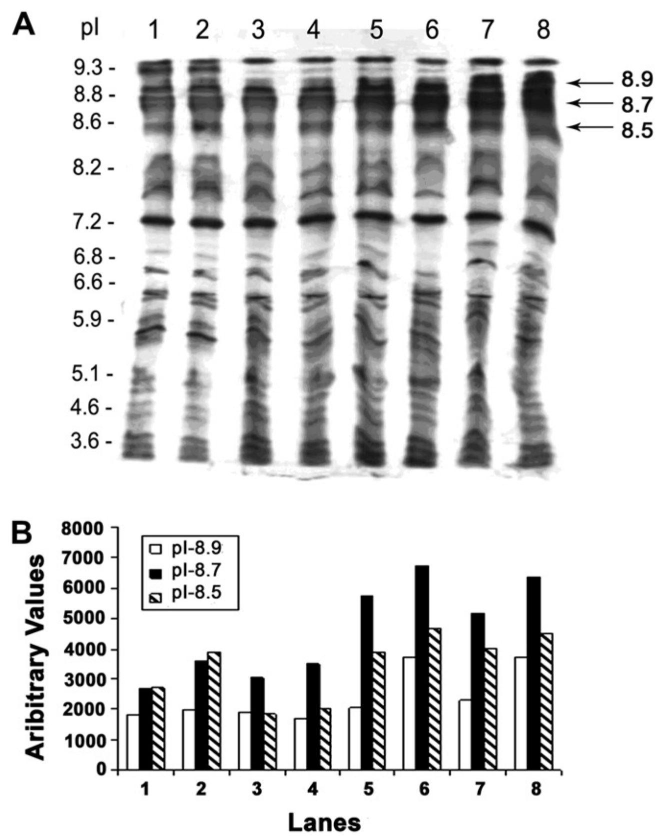

Fig. 6. Peroxidase isoforms (A) Isoelectric focusing of peroxidase isozymes from coleoptile extracts of the resistant cv. IP18296, susceptible cv. 7042S and elicitor treated susceptible pearl millet seedlings. (B) Quantification of the band intensity of three important isoforms (p*I* 8.9, 8.7 and 8.5 respectively) using the Image Analysis System. Lane 1: resistant control; Lane 2: resistant inoculated with *S. graminicola*; Lane 3: susceptible control; Lane 4: susceptible inoculated; Lane 5: chitosan treated susceptible control; Lane 6: chitosan treated susceptible inoculated; Lane 7: *P. fluorescens* treated susceptible control; Lane 8: *P. fluorescens* treated susceptible inoculated. Prominent isozymes with differential expression are indicated (6A).

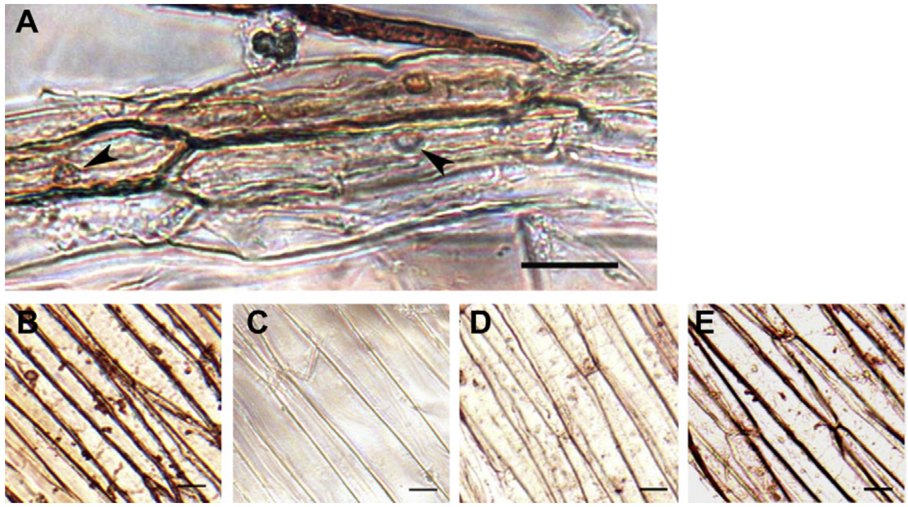

Fig. 7. Accumulation of H_2O_2. (A) The accumulation of H_2O_2 was observed as brownish-black spots within cells close to the parasite haustoria (arrowheads) undergoing a hypersensitive–like reaction in resistant pearl millet. The pattern of H_2O_2 accumulation following staining with DAB was also observed in cell wall peelings. The samples are (B) Resistant cv. IP18296, 8 hai with *S. graminicola*; (C) Susceptible cv. 7042S; (D) Susceptible cv. 7042S, 8hai with *S. graminicola*; (E) Chitosan treated susceptible seedlings, 8 hai with *S. graminicola*. Bars 50 μm.

elicitors triggers the defense reaction in pearl millet which includes the accumulation of HRGPs in the cell walls.

In the present study, soluble proteins were removed by repeated washes with buffer and water and the insoluble cell wall proteins were extracted from the cell wall by using an acid and ethanol mixture. This process results in a protein preparation that is rich in HRGPs [31]. SDS-PAGE of these acid-ethanol extracted cell wall proteins followed by Coomassie blue staining showed several proteins. Three proteins with molecular weights of 27, 17 and 14 kDa reacted with the MAC 265 monoclonal antibody on western blots. This antibody was originally isolated by [24] based on its recognition of interface proteins in pea-*rhizobium* symbiosis and the antibody has been used earlier to identify HRGPs in legumes [14] beans and soybean [18]. Our recent studies purified a heteromer of Proline/Hydroxyproline rich glycoprotein (P/HRGP) from resistant pearl millet cultivar IP18296. This heteromer is envisaged to disintegrate into monomers, dimers and trimers during acid-ethanol extraction and denaturing SDS-PAGE analysis [32]. The 14 kDa HRGP was observed in highly susceptible pearl millet varieties only upon pathogen inoculation [33]. Interestingly in the present study an induction of 14 kDa HRGP was observed upon treatment of susceptible cultivars with the elicitors of defense. Sensitizing a susceptible plant with a suitable elicitor has been reported to result in more rapid response of the plant against virulent pathogens [34,35]. An increase in the 14 kDa HRGPs observed in the susceptible cultivars during seed priming indicates a protective role for this protein in protection of pearl millet against downy mildew.

Tissue printing followed by immunolabeling of HRGPs using MAC 265 antibody showed a higher accumulation of HRGPs in the regions of vascular bundles of the coleoptiles. An intense accumulation of HRGPs in the resistant cultivar of pearl millet compared to the susceptible one during *S. graminicola* inoculation was recorded. In addition, an increased accumulation of HRGPs was observed in the tissues of susceptible cultivar treated with chitosan. Accumulation of HRGPs in phloem cells of pearl millet may contribute to the defense response designed to prevent systemic spread of the pathogen through the vascular system [36].

HRGPs are thought to be initially synthesized as monomers and following oxidative burst after perceiving the presence of a pathogen, they cross-link with each other through covalent bridges to form an insoluble barrier [37]. The possible mechanism by which HRGP accumulation contributes to disease resistance involves cross-linking between HRGP monomers to form a network which might provide anchorage for lignifications and create a barrier impenetrable to fungal hyphae [16,18]. This might also lead to obstruction of haustoria production and nutrient shortage, which may be particularly unfavorable for biotrophic pathogens [38]. It has also been proposed that HRGPs could act as microbial agglutinins [16].

The HRGP cross-linking is a peroxidase mediated process in the presence of H_2O_2. In our study on the pearl millet- downy mildew interaction, H_2O_2 accumulated to a higher extent in the highly resistant cultivar compared to the highly susceptible cultivar. We found clear indications of HR responses in the cell wall peelings of the resistant cultivar of pearl millet upon *S. graminicola* inoculation at 8 hai. It was observed that HRGPs in the hypersensitive response (HR) cells are cross-linked, a process fuelled by H_2O_2 which limit pathogen entry to other parts of the plant [29]. Our findings of an intense accumulation pattern for H_2O_2 in cells close to the *S. graminicola* haustoria in the resistant variety undergoing HR reactions gives an indication of the possible HRGP cross-linking that can take place in those regions to stop the pathogen ingress.

In the present study, an early accumulation of H_2O_2 by 2 h following inoculation was recorded that continued reaching a peak by 8 hai. It was observed that maximum H_2O_2 accumulation was in the chitosan and *P. fluorescens* treated susceptible cultivar at 8 hai. This higher accumulation of H_2O_2 in elicitor treated pearl millet seedlings at 8 hai coincided with the onset of induction of HRGPs using the elicitors at the same time interval of 8–9 h. Peroxidase activity also followed a similar pattern Treatment with chitosan and *P. fluorescens* of the susceptible cultivar resulted in a marginal increase in peroxidase activity, which increased substantially after challenge inoculation with *S. graminicola*. In other host pathogen interactions for example in barley inoculated with *Blumeria graminis* f.sp. *hordei*, H_2O_2 accumulates several hours before cell death, first subcellularly, directly beneath fungal appressoria; then during a second H_2O_2 burst, filling the entire attacked epidermal cell [29]. Similar results were obtained in case of wheat-powdery mildew interactions [39]. HRGP cross-link in the presence of H_2O_2 and the peroxidase enzyme *in-vitro* [32]. Similar kind of cross-linking is possible *in muro* or inside the plant cell wall after oxidative burst or H_2O_2 accumulation to stop the pathogen ingress [36].

The class III plant peroxidases (Prxs) belonging to the basic isoforms of the superfamily of peroxidases helps in cell wall cross-linking in presence of H_2O_2 [40,41]. In our study, IEF analysis of peroxidase indicated that isoforms with p*I* 8.9, 8.7 and 8.5 were induced in resistant, chitosan and *P. fluorescens* treated seedlings infected with *S. graminicola*. These results corroborate earlier reports where basic isoforms of peroxidase were involved in HRGP cross-linking. A cationic peroxidase with cell wall cross-linking activity was also reported in rice plants infected by *Xanthomonas oryzae* pv. *oryzae* [42]. Jackson et al. [43] reported HRGP cross-linking activity induced by a cationic peroxidase isozyme (p*I* 8.8) in tomato.

In conclusion the results of the present study clearly indicate that the seed treatment of pearl millet with elicitors of downy mildew resistance lead to accumulation of HRGPs. This accumulation may be one of the mechanisms by which elicitors offer resistance. Thus induction of host structural defense against the pathogen offers an interesting alternative for the management of the downy mildew disease.

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